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Chemotaxis of *Aeromonas hydrophila* to the Surface Mucus of Fish

Terry C. Hazen, †* Gerald W. Esch, ‡ Ronald V. Dimock, Jr., ‡ and Anne Mansfield ‡

†Department of Biology, Faculty of Natural Sciences, University of Puerto Rico, Rio Piedras, Puerto Rico 00931 ‡Biology Department, Wake Forest University, Winston-Salem, North Carolina 27109, USA

Abstract. Isolates of Aeromonas hydrophila from various sources show different chemotactic responses to mucus from the surface of freshwater fish. Some isolates were nonchemotactic to fish surface mucus. Isolates of A. hydrophila from fish lesions had a significantly higher chemotactic index than isolates of A. hydrophila from water. Maximum chemotactic responses occurred more often to diluted fish mucus than to undiluted samples. Fish which were experimentally stressed did not produce mucus that was more or less chemotactic than that of unstressed fish. Fish with red-sore lesions produced surface mucus which was not chemotactic to A. hydrophila. Differences between fish, for any isolate, were also not significant. The chemotactic substance(s) in fish mucus has a molecular weight of approximately 100,000 and did not appear to be labile when heated to 56°C.

Aeromonas hydrophila has long been recognized as a pathogen of amphibians [9]; however, recently it has received increased attention as a pathogen of reptiles [22], turtles [25], snail [23], alligators [14]. fish [10,24], cattle [28], and man [5,27]. Commercial and sports fisheries losses to A. hydrophila have become staggering in recent years, e.g., in one North Carolina reservoir more than 37,500 fish died over one 13-day period from red-sore disease [24]. The primary etiological agent of red-sore disease in fish in the southeastern United States is A. hydrophila [20]. Histological and hematological studies of largemouth bass taken from natural populations indicate that the disease begins as a small surface lesion, followed by sloughing of scales, local hemorrhage, and septicemia [21]. Infection does not always result in death but, when it does, damage of the liver and kidneys due to bacterial toxicity is always observed [21]. Spontaneous healing occurs in some fish, with a resultant high serum titer of anti-A. hydrophila, Ig M-like antibody [17]. This anti-A. hydrophila antibody also appears to be protective for no more than one season [17].

Possible modes of red-sore disease infection in fish are: the gastro-intestinal route via ingestion with subsequent foci of infection in peripheral tissues, and/or direct penetration of the scale epithelia through the surface mucus [20,21]. Since A. hydro-

phila is known to form at least six toxins [3,4,6], the microcolony erosion theory of the surface mucosa-epithelia, is a much more tenable hypothesis. One possible method by which these microcolonies may come to be associated with the surface epithelium and, subsequently, to increase sufficiently to cause scale erosion, is chemotaxis. Since A. hydrophila is a Gram-negative rod that normally has a single, polar flagellum [8], chemotactic behavior seems likely. In the present study, we explore the chemoattractant nature of fish mucus to A. hydrophila, elsewhere (T. C. Hazen et al., submitted, Canadian Journal of Microbiology), we discuss the motility and chemotaxis of A. hydrophila to various carbohydrates and amino acids.

Materials and Methods

Collection of fish mucus. Sexually mature largemouth bass (*Micropterus salmoides*), both healthy and infected, were captured by angling and electrofishing at Par Pond, near Aiken, South Carolina; all fish were > 20 cm in total length. Fish were washed with sterile distilled water by repeatedly flushing the surface of the fish with the same 100-ml portion of water. After washing the fish until the solution had become viscous, it was filter sanitized with 0.45- μ m-pore-diameter membrane filter (Millipore Corp., Bedford, Massachusetts) and stored under refrigeration (4°C).

Isolation of bacteria. During the course of environmental investigations on *Aeromonas hydrophila* [13,15,16,20] random, single colonies were selected from count estimates. In all cases, R-S

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medium [26] which has been shown to be more than 94% presumptive for *A. hydrophila* was the primary means for isolating this bacterium. Yellow colonies were selected after 24-h incubation at 35°C. These isolates were further characterized and confirmed as *A. hydrophila* using API-20E (Analytab Products, Plainview, New York), oxidase tests, O/129 sensitivity, serology, and fluorescent antibody [13,20]. Isolates were taken from a variety of sources including water, sediment, fish, alligators, and man. After being completely characterized as *A. hydrophila*, all isolates were grown in carbohydrate-free media, dispensed to small vials with equal volumes of glycerin, and stored at -70°C until used. This method was found to preserve isolates for more than two years (T. C. Hazen, unpublished data).

Chemotaxis assay. The chemotaxis assay was basically a modification of Adler's [1] technique. Isolates were grown in 3% TSB (Trypticase soy broth, BBL Microbiology Systems) for 24 h and harvested by centrifugation at $6,000 \times g$ for 10 min at 4°C. The pellet was resuspended in an equal volume of phosphate-buffered saline (PBS) (pH 7), and centrifuged again; the washing step was repeated twice more. Final resuspension was in chemotactic buffer (KPB) [1], and the cell density was adjusted to 10^9 cells ml^{-1}

The bacterial suspension was dispensed in 0.25-ml quantities to 6 × 12-mm test tubes. A capillary tube, closed at one end and containing the substrate to be tested, was introduced into each test tube [1]. After incubating the capillary tube in the bacterial suspension for 1 h at 35°C (the optimum growth temperature for A. hydrophila [18]), the capillary tubes were removed and the contents washed into a diluting vial containing 10 ml of a sodium azide-free isotonic diluting solution (Fisher Scientific Company, Fairlawn, New Jersey). Cell counts were made directly from the diluting vial, using a model ZF Coulter counter (Coulter Electronics, Hialeah, Florida). Ten replicates were used for every substrate tested. Each substrate was tested at four different dilutions, undiluted to 10^{-2} , and included a KPB control, and a motility test; all dilutions of substrates were done with KPB. The KPB control was done exactly as described above for substrate tests except that the capillary tube was filled with KPB. A motility test was also conducted. It consisted of a KPB control in which 0.01 ml of the test substrate was added directly to the bacterial suspension prior to incubation with the capillary tube containing KPB. Motility is defined as the ability to increase activity in the absence of a specific point source of substrate. Thus, if motility is significant, the motility test counts will exceed the KPB control counts.

Characterization of fish mucus. To test for heat lability, the fish mucus was heated at 56°C for 60 min. Both heated and unheated surface mucus from the same fish were tested for chemotactic activity as described above. Separation of the surface mucus into various molecular weight fractions was accomplished by ultrafiltration. Pellicon membrane filters, 47 mm diameter (Millipore Corp.) were used to separate fish surface mucus into nominal molecular weight fractions (NMW) i.e., PSAC-1,000 NMW, PTGC-10,000 NMW, PSED-25,000 NMW, PTHK-100,000 NMW. Each fraction was simultaneously tested with unfractionated surface mucus from the same fish using the chemotaxis assay described above.

Data analysis. All dilutions of each substrate, the KPB control, and motility test were tested for differences using analysis of variance. All counts were transformed with log [x] before analysis, to reduce heteroscedascity as determined by skew and

kurtosis. Group means found to be significantly different were further differentiated from each other statistically using a Student-Newman-Keuls multiple range test. Any probability less than or equal to 0.05 was considered significant [29].

Results

Fish surface mucus promoted significant chemotaxis by most isolates of Aeromonas hydrophila over the whole range of dilutions tested (Fig. 1). Of the twelve A. hydrophila isolates tested, nine were positively chemotactic to fish surface mucus (Table 1). Isolates of A. hydrophila from fish lesions exhibited a significantly greater chemotaxis than did isolates from water (chemotaxis indices = 1.31, n = 5 and 1.13, n = 8, respectively). Higher responses were also observed at low dilutions of fish mucus, as compared to the responses to undiluted mucus. The motility test was not significant for any isolates. Each isolate of A. hydrophila responded in the same way to surface mucus from different fish.

The response of A. hydrophila to heated mucus and to mucus from fish treated with cortisol was not significantly different (Table 2). However, surface mucus from infected fish failed to elicit a significant chemotactic response. Only the fractions of surface mucus which were near or greater than 100,000 NMW induced significant chemotaxis in A. hydrophila.

Discussion

The chemotactic responses of Aeromonas hydrophila to fish mucus are of similar magnitude as responses observed for carbohydrates and amino acids (T. C. Hazen et al., submitted). Higher accumulations observed in diluted mucus could be due to differences in viscosity that would allow greater bacterial movement. However, some of the isolates tested failed to show a significant chemotactic response to fish surface mucus, and the motility test was not significant for any of the isolates. Thus, those isolates which responded to the mucus did so by chemotaxis, not simply by a kinetic response involving a change in motility. Bacterial chemotaxis to intestinal mucosal surfaces has been reported before [2]; however, this is a first report for fish surface mucus.

The differential responses of isolates of A. hydrophila to fish surface mucus, and the larger chemotactic index of isolates of A. hydrophila from fish lesions, suggest the existence in nature of certain "strains" of A. hydrophila which are more likely to cause fish disease. Studies of cross-reactiv-

Table 1. Chemotactic index of *Micropterus salmoides* surface mucus for different isolates.^a

Source of isolate	Concentration (dilution ratio)						
	Un- diluted	1:2	1:10	1:100	Motility		
Fish lesion (LN1)	1.39	1.80	1.53	1.10	1.08		
Fish lesion (CR10)	0.97	0.87	0.91	1.05	0.91		
Fish lesion (BLMB23)	1.43	1.18	0.91	0.90	0.71		
Fish lesion (BSM)	1.46	1.25	1.05	0.95	0.90		
Fish lesion (849L)	1.28	1.01	1.34	1.49	1.01		
Reservoir water (LNH)	0.85	0.84	0.85	0.89	0.89		
Lake water (33A)	1.12	1.25	0.97	0.96	0.92		
River water (MR)	$\overline{1.28}$	1.03	1.15	1.09	0.91		
River water (BR)	1.48	1.30	1.10	0.87	0.79		
Lake water (FT4)	0.88	0.83	0.80	0.91	1.01		
Lake water (HDW)	1.25	1.22	1.18	1.12	0.91		
Lake water (FT1)	$\overline{1.03}$	0.98	0.94	1.01	0.81		
Reservoir water (HR)	<u>1.14</u>	0.84	1.08	0.95	0.91		

^a All values are the mean of 10 determinations; standard deviations of the mean were always less than 0.14. Underlined values are significant as determined by analysis of variance. Chemotactic index = experimental cell count/control cell count. For details of isolation sites, see Hazen et al. [17,19]. Dilutions = concentrate:final volume.

Table 2. Chemotactic index of $Micropterus\ salmoides\ surface$ mucus.^a

Type of mucus	Concentration (dilution ratio)						
	Un- diluted	1:2	1:10	1:100	Motility		
Normal	1.81	1.40	1.27	1.13	0.93		
Heated (56°C for 60 min)	$\overline{1.71}$	1.32	1.25	1.05	0.96		
Cortisol (200 μ g kg ⁻¹)	1.40	1.25	1.21	1.09	0.91		
Red-sore infected fish	0.90	0.97	0.82	0.97	0.89		
<10,000 NMW	0.77	0.70	0.60	0.59	0.51		
>10,000 NMW	1.12	1.14	0.66	0.67	0.55		
10,000-100,000 NMW	0.89	0.80	0.96	0.85	0.90		
<100,000 NMW	1.58	0.69	0.65	0.80	0.81		
>100,000 NMW	0.76	1.12	1.13	1.04	0.78		

^a All values are the mean of 10 determinations; standard deviations of the mean were always less than 0.15. Underlined values are significant as determined by analysis of variance. NMW = nominal molecular weight. Chemotactic index = experimental cell counts/control cell counts. Dilutions = concentrate:final volume.

ity of antisera from largemouth bass from different reservoirs with A. hydrophila isolates from those same reservoirs show that the best reactions were in homologous systems [17], suggesting the potential for differential virulence among various strains. This could create conditions conducive for local epizootics, a phenomenon that has been reported previously [17,25].

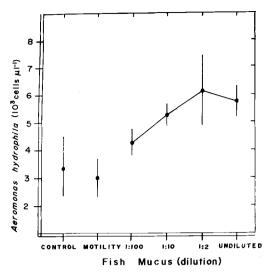


Fig. 1. Typical chemotactic response curve for *Aeromonas hydrophila* to diluted and undiluted fish mucus. All points are the mean of 10 determinations with bars representing one standard deviation from the mean. Dilutions = concentrate:final volume.

The response of a particular A. hydrophila isolate to surface mucus from different fish was not significantly different, but only if the fish were healthy. On the other hand, surface mucus from fish with red-sore disease was not significantly chemoattractant to any isolate of A. hydrophila. Indeed, surface mucus from infected fish not only failed to elicit chemotaxis, it was repellant (Table 2).

Other studies have shown that infected largemouth bass develop significant anti-A. hydrophila antibody titers [17]. A possible mechanism for the repellant nature of surface mucus from infected fish may be the excretion of anti-A. hydrophila antibodies into surface mucus. Excretion of antibodies into surface mucus is well documented in fish [12]. Largemouth bass with high titers to anti-A. hydrophila antibodies are apparently protected from A. hydrophila infection for at least one season [17]. This protection is further supported by the observation that the bacteria remained viable (plate counts) when incubated with the mucus from infected fish, thus, eliminating the possibility of killing as the reason for lower accumulations. The protection then may largely be the result of the repellant nature of the fish mucus.

Largemouth bass which are under stress have significantly greater red-sore disease incidence than

nonstressed fish [10.11]. Others have observed that aquatic animals under stress produce significantly more surface mucus than nonstressed animals [7]. Therefore, largemouth bass were injected with cortisol to induce stress and determine if qualitative changes occurred in the mucus that might increase chemotaxis. The surface mucus from fish which were given weekly injections of cortisol (200 mg kg^{-1}) for one month [11] was similar in chemoattraction to that of nonstressed fish. It would appear, therefore, that changes in the chemoattractant properties of fish surface mucus caused by acute stress probably do not influence the observed correlation between stress and the prevalence of red-sore disease infection rate in fish. However, long-term or chronic stress effects on fish surface mucus cannot be ruled out [11].

The substance(s) in fish surface mucus which induces chemotaxis by A. hydrophila is insensitive to heat and has a molecular weight near or in excess of 100,000, but is not equally attractive to all isolates. Dialyzed mucus did not give a significantly different response from undialyzed mucus, thus eliminating the possibility that small molecules which might interact with the mucus could be eliciting chemotaxis. The attractive properties of fish mucus are in some way modified or masked in fish infected with red-sore disease. Inoculation of cortisol does not alter the release of chemotactic substances within the surface mucus. It is suggested, therefore, that chemotactic substance(s) in fish surface mucus may stimulate infection by A. hydrophila and that masking of the substance(s) may protect fish from acquiring red-sore disease.

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